PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C07K 13/00, C12P 21/02
C12N 15/00, A61K 37/02

A1

(11) International Publication Number: WO 90/11301

(43) International Publication Date: 4 October 1990 (04.10.90)

(21) International Application Number: PCT/US90/01355

(22) International Filing Date: 15 March 1990 (15.03.90)

(30) Priority data:
325,408 17 March 1989 (17.03.89) US

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(81) Designated States: AT (European patent), BE (European + patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: HUMAN MACROPHAGE MIGRATION INHIBITORY FACTOR

(57) Abstract

This invention provides a novel human macrophage migration inhibitory factor and processes for obtaining this factor in homogeneous form and producing it by recombinant genetic engineering techniques.

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HUMAN MACROPHAGE MIGRATION INHIBITORY FACTOR

The present invention relates generally to a novel protein factor which is important in controlling a variety of inflammatory responses. More specifically the invention discloses a novel human macrophage migration inhibitory factor. Also provided are processes for obtaining this factor in homogeneous form and producing it by recombinant genetic engineering techniques.

Background of the Invention

In response to antigenic or mitogenic stimulation, lymphocytes secrete protein mediators called lymphokines that play an important role in immunoregulation, inflammation and effector mechanisms of cellular immunity [S. Cohen et al, "Biology of the Lymphokines", New York, Academic Press, pp. 511-576 (1979); and A. Miyajima et al, FASEB J., 38:2462-2473 (1988)]. The first reported lymphokine activity was observed in culture supernatants of antigenically sensitized and activated guinea pig lymphocytes. This activity was named migration inhibitory factor (MIF) for its ability to prevent the migration of guinea pig macrophages out of capillary tubes in vitro [B. R. Bloom, et al, Science, 153:80-82 (1966); and J. R. David, Proc. Natl. Acad. Sci., 153:72-77 (1966)].

Since this initial observation of MIF activity, a large number of publications have reported the isolation and identification of putative MIF molecules. See. e.g., P. S. Papageorgiou et al, J. Immunol., 108:494-504 (1972); R. E. Rocklin et al, Cell. Immunol., 5:435 (1972); T. Yoshida et al, J. Immunol., 117:548-554 (1976); H. G. Remold et al, J. Immunol., 118:2015-2019 (1977); L. H. Block et al, J. Exp. Med., 147:541-553 (1978); G. Pozzanza et al, Science, 205:300-301 (1979); P. N. Dinh et al, J. Interferon Res., 1:23 (1981); W. Y. Weiser et al, J. Immunol., 126:1958-1962 (1981); S. Z. Salahuddin et al, Science, 223:703-707 (1984); W. Y. Weiser et al, Cell. Immunol., 88:109-122 (1984); W. Y. Weiser et al, Cell. Immunol., 93:532-540 (1985); D. T. Umetsu et al, J. Immunol., 140:4211-4216 (1988) and G. Zwadlo et al, Clin. Exp. Immunol., 72:510-515 (1988).

However, other lymphokines, e.g., interferon gamma and IL-4, which exhibit MIF activity, among other activities, have only recently been identified [G. B. Thurman et al, J. Immunol., 134:305-309 (1985); and A. McInnes et al, J. Exp. Med., 167:598-611 (1988)]. These observations that other known lymphokines have 'MIF' activity have raised considerable doubt that distinct novel entities with MIF activity exist.

The detection of MIF activity is correlated with a variety of inflammatory responses including delayed hypersensitivity and cellular immunity [R. E. Rocklin et al, New Engl. J. Med., 282:1340-1343 (1970); and J. R. David et al, Progr. in Allerg. Immunol., 16:300-449 (1972)]; allograft rejection [S. Al-Askari et al, Nature, 205:916-917 (1965); and J. T. Harrington, Cell. Immunol., 30:261-271 (1977)]; and rheumatoid polyarthritic synovialis [Odink et al, Nature, 330:80-82 (1987)].

There remains a need in the art for biologically active proteins, such as MIF, that influence macrophage function for preparing pharmaceutical compositions useful in stimulating host defense.

Summary of the Invention

In one aspect the present invention provides a novel human macrophage migration inhibitory factor (MIF) which is substantially free from association with other mammalian proteins. This protein is produced by recombinant genetic engineering techniques. The biologically active MIF protein of this invention is comprised of an approximately 115 amino acid sequence. Its amino acid sequence is identified below.

Active MIF has an apparent molecular weight of approximately 12 kd as determined by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) of supernatant fluid derived from MIF cDNA transfected COS-1 cells.

The MIF protein of this invention has displayed biological activities in various assays, which indicate its role as a general activator of several different macrophage functions. The MIF of this invention inhibits the migration of human macrophages in an assay using human peripheral blood cells. MIF displays biological activity in this assay of greater than 20% inhibition. MIF also serves to stimulate the activity of macrophages in culture measured by several different criteria. MIF-treated macrophages express higher than normal levels of IL-1β and HLA-DR mRNA. MIF alone also stimulates macrophages to kill the intracellular pathogen, Leishmania donovani, and also enhances the effects of interferon-gamma in this system.

Another aspect of the present invention is a DNA sequence coding on expression for human MIF protein. The DNA sequence coding for active MIF is characterized as comprising the same or substantially the same nucleotide sequence in Table 1 or fragments thereof.

The MIF DNA sequence of the present invention encodes a polypeptide of 115 amino acids which corresponds well with the molecular weight of a novel protein band revealed by SDS-PAGE of supernatant fluid derived from the same MIF-cDNA transfected COS-1 cells. When this protein band was excised from the gel and electroeluted, it showed strong MIF activity. Analysis of RNA extracted from Con A-stimulated and unstimulated human peripheral blood lymphocytes showed that this cDNA hybridized to a single mRNA species from stimulated but not unstimulated lymphocytes.

Also provided by the present invention is a vector containing a DNA sequence encoding MIF in operative association with an expression control sequence. Host cells transformed with such vectors for use in producing recombinant MIF are also provided by the present invention.

The vectors and transformed cells of the invention are employed in another aspect, a novel process for producing recombinant human MIF protein, or peptide fragments thereof. In this process a cell line transformed with a DNA sequence encoding on expression MIF protein or a peptide fragment thereof in operative association with a suitable expression control sequence capable of controlling expression of the protein is

cultured. This claimed process may employ a number of known cells as host cells for expression of the protein. Presently preferred cell lines for preparing MIF are mammalian cell lines and bacterial cells.

Another aspect of this invention provides pharmaceutical compositions containing a therapeutically effective amount of recombinant MIF or of one or more peptide fragments thereof. These pharmaceutical compositions may be employed in methods for treating disease states or injuries in which the activation of macrophages plays a key role. For example, MIF protein or active fragments thereof may be employed in therapies for cancer, the treatment of infections, acceleration of wound healing and in stimulating the immune system in general. MIF may also be used in potentiating the immune response to certain antigens, particularly vaccines.

A further aspect of the invention, therefore, is a method for treating these and/or other pathological states by administering to a patient a therapeutically effective amount of MIF or peptide fragments thereof in a suitable pharmaceutical carrier. These therapeutic methods may include administering simultaneously or sequentially with MIF or peptide fragments thereof an effective amount of at least one other cytokine,

hematopoietin, interleukin, growth factor, or tumor specific antibody.

Other aspects and advantages of the present invention are described further in the following detailed description of preferred embodiments of the present invention.

Detailed Description of the Invention

The present invention provides a biologically active human macrophage migration inhibitory factor (MIF) in a form substantially free from association with other mammalian proteins. This protein can be produced via recombinant techniques to enable large quantity production of pure, active MIF useful for therapeutic applications.

The active human MIF of this invention is characterized by an approximately 115 amino acid protein sequence illustrated in Table I below. MIF has an apparent molecular weight of approximately 12 kd, as determined by SDS-PAGE under reducing conditions.

The DNA sequence of human MIF was initially cloned from a cDNA library prepared from mRNA derived from a phytohemagglutinin and PMA-stimulated human T-cell hybridoma line, T-CEMB [available upon request from W. Weiser, Brigham and Women's Hospital] according to the expression cloning method previously described in, e.g.,

G. G. Wong et al, Science, 228:810-815 (1985); Y. C. Yang et al, Cell, 47:3-10 (1986); and A. E. Namen et al, Nature, 333:571-573 (1988). The library was constructed in an expression vector which permits the expression of cDNA inserts in mammalian cells, e.g. COS-1 cells. Screening of the library was performed by transfecting COS-1 cells with pools of cDNA clones. By assaying the supernatant fluid for MIF activity, cDNA clones expressing MIF activity were identified.

mRNA from several cellular sources was examined for its ability to hybridize with a selected MIF cDNA clone. Northern blot analysis revealed that the T-cell line, CEM, and the T-cell hybridoma line, T-CEMB, as well as lectin-stimulated human peripheral blood lymphocytes (PBL) synthesized readily detectable levels of mRNA that hybridized with the MIF clone. The message, however, could not be detected in RNA samples from unstimulated PBL despite prolonged exposure. The presence of RNA transcript in activated human PBL suggests that the human MIF gene is expressed as a product of activated lymphocytes.

The MIF cDNA sequence from this clone, shown in Table I below, encodes the 115 amino acid sequence (single letter code).

Table I

```
          10                  30                  50
          .                   .                   .
CTCGAGCTGCAGAGCTGCCTCTGCGCGGGTCTCCTGGTCCTTCTGCCATCATGCCGATGT
                                                  M  P  M  F

          70                  90                  110
          .                   .                   .
TCATCGTAAACACCAACGTGCCCCGCGCCTCCGTGCCGGACGGGTTCCTCTCCGAGCTCA
  I  V  N  T  N  V  P  R  A  S  V  P  D  G  F  L  S  E  L  T

         130                 150                 170
          .                   .                   .
CCCAGCAGCTGGCGCAGGCCACCGGCAAGCCCCCCCAGTACATCGCGGTGCACGTGGTCC
  Q  Q  L  A  Q  A  T  G  K  P  P  Q  Y  I  A  V  H  V  V  P

         190                 210                 230
          .                   .                   .
CGGACCAGCTCATGGCCTTCGGCGGCTCCAGCGAGCCGTGCGCGCTCTGCAGCCTGCACA
  D  Q  L  M  A  F  G  G  S  S  E  P  C  A  L  C  S  L  H  S

         250                 270                 290
          .                   .                   .
GCATCGGCAAGATCGGCGGCGCGCAGAACCGCTCCTACAGCAAGCTGCTGTGCGGCCTGC
  I  G  K  I  G  G  A  Q  N  R  S  Y  S  K  L  L  C  G  L  L

         310                 330                 350
          .                   .                   .
TGGCCGAGCGCCTGCGCATCAGCCCGGACAGGGTCTACATCAACTATTACGACATGAACG
  A  E  R  L  R  I  S  P  D  R  V  Y  I  N  Y  Y  D  M  N  A

         370                 390                 410
          .                   .                   .
CGGCCAGTGTGGGCTGGAACAACTCCACCTTCGCCTAAGAGCCGCAGGGACCCACGCTGT
  A  S  V  G  W  N  N  S  T  F  A  *

         430                 450                 470
          .                   .                   .
CTGCGCTGGCTCCACCCGGGAACCCGCCGCACGCTGTGTTCTAGGCCCGCCCACCCCAAC

         490                 510
          .                   .
CTTCTGGTGGGGAGAAATAAACGGTTTAGAGACAGCTCTGCAG
```

The cDNA sequence of Table I contains a long open reading frame of 345 nucleotides, beginning with an ATG codon at nucleotides 51-53. The ATG is followed 114 codons and a TAA termination triplet at nucleotides 396-398. The 345 nucleotides encodes a 115 amino acid polypeptide with a calculated molecular mass of 12,540 which is in agreement with the mass of the MIF-specific protein band observed by pulse-labeling experiments.

Although MIF has been thought to be a secreted protein, the DNA sequence does not contain a stretch of hydrophobic amino acids that resemble conventional secretory leader sequences [D. Perlman et al, J. Mol. Biol., 167:391-409 (1983)], either at the N-terminus or internally. The lack of a very hydrophobic sequence that is characteristic of a protein signal peptide is also observed in the MIF related proteins [K. Odink et al, cited above]. The apparent absence of a leader sequence suggests that the mechanism of MIF secretion is distinct from that of typical secretory proteins. It is possible that this MIF may not be actively or efficiently secreted. Alternatively, cells may export MIF by a mechanism similar to that of IL-1.

The cDNA sequence for MIF also encodes two potential asparagine-linked glycosylation sites at amino acids 73-75 (Asn-Arg-Ser) and 110-112 (Asn-Asn-Ser) [see,

e.g., R. J. Winzler, "The Chemistry of Glycoproteins in Hormonal Proteins and Peptides", Vol. 1, C. H. Li, ed. Academic Press, New York, pp. 1 (1973)]. Like beta-interferon and IL-2, the MIF DNA sequence encodes three cysteine residues, located at amino acid positions 56, 59, and 80. These three cysteine residues may account, at least in part, for the loss of MIF biological activity upon storage.

The nucleotide sequence of this MIF cDNA of the invention has been compared with the nucleotide sequences recorded in Genbank. No significant similarities in nucleotide sequence were found. Of note, human MIF shares no sequence similarity with gamma interferon or IL-4, other cytokines with MIF activity. In addition, no sequence similarity exists between the MIF cDNA of this invention and the cDNAs encoding two proteins, MRP-8 and MRP-14 as reported by Odink et al, cited above. Thus MIF of this invention is immunologically distinct from these known factors and proteins.

The cDNA sequences of the present invention encode biologically active human MIF by detection of the functional polypeptides produced by mammalian cells. One cloned sequence in plasmid p7-1 was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland on March 17, 1989 under No. 40582. This deposit meets the requirements of the Budapest Treaty on the International Recognition of the deposit of

Microorganisms for the Purposes of Patent Procedure and the Regulations thereunder (Budapest Treaty).

The present invention also encompasses these novel DNA sequences, free of association with DNA sequences encoding other primate proteins, and coding on expression for MIF polypeptides. These DNA sequences include those containing the same or substantially the same sequence as the above-identified DNA and peptide sequences and those sequences which hybridize under stringent hybridization conditions [see, T. Maniatis et al, Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory (1982), pages 387 to 389] to the DNA sequence of MIF reported above. An example of one such stringent hybridization condition is hybridization at 4XSSC at 65°C, followed by a washing in 0.1XSSC at 65°C for an hour. Alternatively an exemplary stringent hybridization condition is 50% formamide, 4XSSC at 42°C.

DNA sequences which hybridize to the sequences for MIF or active fragments thereof under relaxed hybridization conditions and which code on expression for MIF peptides having MIF biological properties also encode novel MIF polypeptides. Examples of such non-stringent hybridization conditions are 4XSSC at 50°C or hybridization with 30-40% formamide at 42°C. For example, a DNA sequence which shares regions of significant homology, e.g., sites of glycosylation or

disulfide linkages, with the sequences of MIF and encodes a protein having one or more MIF biological properties clearly encodes a MIF polypeptide even if such a DNA sequence would not stringently hybridize to the MIF sequences.

Similarly, DNA sequences which code for MIF polypeptides coded for by the sequence of MIF, but which differ in codon sequence due to the degeneracies of the genetic code are also encompassed by this invention. Allelic variations (naturally-occurring base changes in the species population which may or may not result in an amino acid change) DNA sequences encoding the MIF protein sequences and peptide fragments thereof evidencing MIF biological activity are also included in the present invention as well as analogs or derivatives thereof. Other variations in the DNA sequence of MIF which are caused by point mutations or by induced modifications to enhance the activity, half-life or production of the polypeptides encoded thereby are also encompassed in the invention.

MIF polypeptides may also be produced by known conventional chemical synthesis. Methods for constructing the polypeptides of the present invention by synthetic means are known to those of skill in the art.

The synthetically-constructed MIF polypeptide sequences, by virtue of sharing primary, secondary, or tertiary structural and conformational characteristics with MIF polypeptides may possess MIF biological properties in common therewith. Thus, they may be employed as biologically active or immunological substitutes for natural, purified MIF polypeptides in therapeutic and immunological processes.

Modifications in the peptides or DNA sequences can be made by one skilled in the art using known techniques. Modifications of interest in the MIF sequences may include the replacement, insertion or deletion of a selected amino acid residue in the coding sequences. Mutagenic techniques for such replacement, insertion or deletion are well known to one skilled in the art. [See, e.g., United States patent 4,518,584.]

Other specific mutations of the sequences of the MIF polypeptide described herein may involve modifications of one or more glycosylation site. The absence of glycosylation or only partial glycosylation results from amino acid substitution or deletion at the asparagine-linked glycosylation recognition sites or at any site of the molecule that is modified by addition of O-linked carbohydrate. An asparagine-linked glycosylation recognition site comprises a tripeptide

sequence which is specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either asparagine-X-threonine or asparagine-X-serine, where X is usually any amino acid.

A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) results in non-glycosylation at the modified tripeptide sequence. Expression of such altered nucleotide sequences produces variants which are not glycosylated at that site.

Other analogs and derivatives of the sequence of MIF which would be expected to retain MIF activity in whole or in part may also be easily made by one of skill in the art given the disclosures herein. One such modification may be the attachment of polyethylene glycol onto existing lysine residues in the MIF sequence or the insertion of a lysine residue into the sequence by conventional techniques to enable the attachment. Such modifications are believed to be encompassed by this invention.

The present invention also provides a method for producing MIF polypeptides. The method of the present invention involves culturing a suitable cell or cell line, which has been transformed with a DNA sequence

coding on expression for an MIF polypeptide or an active fragment thereof, under the control of known regulatory sequences. Suitable cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO) or 3T3 cells. The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. See, e.g., Gething and Sambrook, Nature, 293:620-625 (1981), or alternatively, Kaufman et al, Mol. Cell. Biol., 5(7):1750-1759 (1985) or Howley et al, U. S. Patent 4,419,446. Other suitable mammalian cell lines, are the monkey COS-1 cell line, and the CV-1 cell line.

Similarly useful as host cells suitable for the present invention are bacterial cells. For example, the various strains of E. coli (e.g., HB101, MC1061 and strains used in the following examples) are well-known as host cells in the field of biotechnology. Various strains of B. subtilis, Pseudomonas, other bacilli and the like may also be employed in this method.

Many strains of yeast cells known to those skilled in the art are also available as host cells for expression of the polypeptides of the present invention.

Additionally, where desired, insect cells may be utilized as host cells in the method of the present invention. See, e.g. Miller et al, Genetic Engineering, 8:277-298 (Plenum Press 1986) and references cited therein.

The present invention also provides vectors for use in the method of expression of novel MIF polypeptides. These vectors contain the novel MIF DNA sequences which code for MIF polypeptides of the invention. Alternatively, vectors incorporating modified sequences as described above are also embodiments of the present invention and useful in the production of MIF polypeptides. The vector employed in the method also contains selected regulatory sequences in operative association with the DNA coding sequences of the invention and capable of directing the replication and expression thereof in selected host cells.

Thus, MIF, produced recombinantly or synthetically, may be used in a pharmaceutical preparation to treat diseases that may be responsive to macrophage activation. It may also be possible to employ an active peptide fragment of MIF in such pharmaceutical formulations. One therapeutic use for pharmaceutical compositions containing the MIF or fragment thereof is in the treatment of cancer. For example, activated macrophages alone or in combination with specific anti-

tumor monoclonal antibodies have considerable tumoricidal capacity. MIF's ability to activate macrophages indicate its use alone or in combination with other therapeutic agents as a potent anti-tumor agent for treatment of cancer patients.

Similarly, the ability of MIF to promote macrophage-mediated killing of certain pathogens indicates the use of this molecule in treating various infections by a number of pathogens including, for example, Leishmania donovani.

In addition, the ability of MIF to prevent the migration of macrophages may be exploited in a therapeutic agent for treating wounds. Local application of MIF protein at the site of injury may result in increased numbers of activated macrophages concentrated within the wound, thereby increasing the rate of healing of the wound.

In addition, MIF may be used as a general immune stimulus and, in particular, may be used to increase the immunity generated against specific vaccines. The ability of MIF to enhance macrophage IL-1β and HLA-DR expression indicates that this molecule enhances the ability of macrophages to present antigens to T cells. Therefore MIF has utility in potentiating the immune response to different antigens. This property

of MIF indicates its usefulness as a general immune stimulus. More particularly, MIF has utility in potentiating the immune response to particular vaccines. This is extremely important in cases, such as the human immunodeficiency viruses, where vaccine development has been particularly problematic.

The MIF protein or fragments thereof of this invention may also be employed, alone or in combination with other cytokines, hematopoietins, interleukins, growth factors, interferons or antibodies to treat a variety of infections, cancer, and perhaps tissue injuries, as described above. In particular co-administration of MIF with IFN gamma, M-CSF or GM-CSF is expected to provide enhanced therapeutic benefit for the conditions described above.

Other uses for these novel polypeptides are in the development of monoclonal and polyclonal antibodies generated by standard methods for diagnostic or therapeutic use.

Therefore, as yet another aspect of the invention are methods and therapeutic compositions for treating the conditions referred to above. Such compositions comprise a therapeutically effective amount of the MIF protein or therapeutically effective fragment thereof of the present invention in admixture with a

pharmaceutically acceptable carrier. This composition can be systemically administered parenterally. Alternatively, the composition may be administered intravenously. If desirable, the composition may be administered subcutaneously. For use in tissue healing, the MIF of this invention would be in a pharmaceutical preparation suitable for local or topical application.

When systematically administered, the therapeutic composition for use in this invention is in the form of a pyrogen-free, parenterally acceptable aqueous solution. The preparation of such a pharmaceutically acceptable protein solution, having due regard to pH, isotonicity, stability and the like, is within the skill of the art.

The dosage regimen involved in a method for treating the above-described conditions will be determined by the attending physician considering various factors which modify the action of drugs, e.g. the condition, body weight, sex and diet of the patient, the severity of any injury or infection, time of administration and other clinical factors. Generally, the daily regimen should be in the range of 1-1000 micrograms of MIF protein or 50 to 5000 units (i.e., one unit per ml being the concentration of protein which

leads to half maximal inhibition in the MIF assay) of protein per kilogram of body weight.

The therapeutic method and compositions of the present invention may also include co-administration with other human factors. Exemplary cytokines or hematopoietins for such use include the known factors IL-1, IL-2, IL-3, IL-4, IL-6, G-CSF, CSF-1, GM-CSF, M-CSF, the interferons, or erythropoietin. More particularly, MIF in combination with IFN gamma, or M-CSF or GM-CSF is expected to provide enhanced therapeutic activity for treatment of the conditions described above. The dosage recited above would be adjusted to compensate for such additional components in the therapeutic composition. Progress of the treated patient can be monitored by conventional methods.

The following examples illustratively describe the cloning, expression and production of human MIF and other methods and products of the present invention. These examples are for illustration and do not limit the scope of the present invention.

Example 1 - Isolation of mRNA and Construction of cDNA Library

A human T-cell hybridoma line, T-CEMB, found to elaborate significant MIF activity but barely detectable amounts of IFN-gamma message, was chosen as the source of

RNA extraction. This cell line was generated by fusion of an HAT-sensitive T-lymphoblastoid line CEMWH4 with Con A-stimulated human peripheral blood T-cells according to W. Y. Weiser et al, Cell. Immunol., 90:167-178 (1985). The cells are maintained in medium consisting of RPMI 1640, 10% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine and 50 ug/ml Gentamicin. Total RNA was extracted according to the method of Chirgwin et al, Biochemistry, 18:5294-5299 (1979) from T-CEMB cells that have been stimulated with PHA (1%) and PMA (10 ng/ml) for 18 hours.

mRNA was prepared by oligo(dT)-cellulose chromatography [H. Aviv et al, Proc. Natl. Acad. Sci. USA, 69:1408-1412 (1972)]. Five micrograms of mRNA was used to synthesize double-stranded cDNA as described by Wong et al, cited above, with DNA polymerase I in the second strand reaction and removal of the hairpin loop by SI nuclease digest [T. Maniatis et al, cited above]. The double-stranded DNA was blunted and ligated to 5-fold excess of synthetic semi-Xho adapters [Yang et al, Cell, 47:3-10 (1986)]. The semi-Xho adapted cDNA was size fractionated by agarose gel electrophoresis. The region of the gel containing cDNA larger than 500 base pairs was excised. These semi-Xho adapted cDNA fragments were isolated by adherence to glass powder [B. Vogelstein et

al, Proc. Natl. Acad. Sci. USA, 76:615-619 (1979)] and subsequent elution with low salt buffer [5 mM Tris, 0.5 mM ethylene diaminetetraacetic acid (EDTA)].

The COS-1 cell expression vector pXM [Y. C. Yang et al, cited above] was linearized at the unique Xho I site and ligated to equimolar amounts of the semi-Xho adapted cDNA. The ligation reaction was used to transform competent Escherichia coli strain HB101 [Y. C. Yang et al, cited above] to generate a library of approximate 60,000 ampicillin-resistant colonies.

Example 2 - DNA Preparation and COS-1 Cell Transfection

The expression cloning system previously described by G. G. Wong et al, cited above, was employed to isolate a cDNA encoding the MIF activity as follows.

Bacterial colonies from the above-described cDNA library were replicated onto nitrocellulose filters. Colonies from each filter were scraped into L-broth and plasmid DNA was isolated by previously described methods [J. A. Meyers et al, J. Bacteriol., 127:1529-1536 (1976)]. Each primary DNA sample was prepared from a pool of 200-500 colonies.

Five micrograms of each plasmid DNA was used to transfect COS-1 cells by DEAE-dextran-mediated DNA transfection, with the addition of a 0.1 mM chloroquine treatment [L. M. Sompayrac et al, Proc. Natl. Acad. Sci.

USA, 78:7575-7578 (1981) and H. Luthman et al, Nucl. Acids Res., 11:1295-1308 (1983)]. Culture supernatant fluid from transfected COS-1 cells was harvested 72 hours after transfection and assayed for MIF activity according to the assay described below in Example 7.

Plasmid DNA from the positive pools was re-transfected into COS-1 cells and transfected supernatants were re-screened for MIF activity. To minimize the chance of false positives, each sample was tested at least five times using cells from different blood donors. These samples were then subdivided to contain fewer clones until individual clones were isolated. Of the 100 supernatants for the initial COS-1 cell transfections of the primary pools, two samples showed the best overall MIF activity.

The pools with the highest MIF activity were selected and subdivided to contain fewer number of clones, their DNAs were prepared, transfected, and the transfected supernatants were examined for MIF activity until single clones expressing MIF activity were obtained.

One clone which consistently demonstrated the best MIF activity was re-examined in the MIF assay of Example 7. The biological activity of this clone was also examined with peritoneal macrophages of guinea pig

and mouse. Inhibition of 38% and 31% was found respectively from a 5-fold dilution of the crude supernatant of transfected COS-1 cells. The MIF activity from this clone was also compared with other cytokines (IL-2, TNF-α, TNF-β, IL-3 and IFN-gamma). The MIF supernatant showed greater MIF activity in a bioassay than all of these cytokines. However, fairly strong MIF activity was displayed by TNF-α and IFN-gamma.

Example 3 - Protein Analysis

The polypeptide encoded by the cDNA of p7-1 was identified using pulse-labeling experiments. SDS-PAGE of proteins secreted by COS-1 cells transfected with p7-1 DNA revealed the presence of a 12 kd polypeptide which was absent in a mock transfected control. This novel band was excised from the polyacrylamide gel and electroeluted at six watts for two hours in elution buffer containing 50 mM NH_4HCO_3 and 200 ng/ml of human serum albumin. The latter was added to prevent nonspecific sticking. As controls, gels with the same molecular weight from mock-transfected supernatant were also excised and subjected to electroelution. The eluant was reconstituted with medium and examined for MIF activity.

Strong MIF activity was found in the eluant. However, no MIF activity was detected in mock gels excised from the same molecular weight region. When bands with 30 kd were removed from the same gels containing active MIF-polypeptide and subjected to electroelution, the eluants from the 30 kd band enhanced migration. This 30 kd protein could be an inhibitor of MIF which antagonizes and/or decreases MIF activity in the crude supernatant.

Forty-eight hours after chloroquine treatment, culture supernatant from COS-1 cells transfected with recombinant DNA of MIF-positive clones was removed and cells were pulse-labelled with 0.5 mCi [35S]methionine in 0.5 ml of DMEM for 4 hours at 37°C. Radiolabelled supernatant was collected and subjected to a 15% SDS-PAGE [U. K. Laemmli, Nature, 227:680-685 (1970)]. After electrophoresis, the gel was immersed in a fluorography enhancing solution (Enhance; New England Nuclear, Boston, MA), dried, and exposed to X-ray film.

Example 4 - RNA Analysis

Twenty micrograms of total cellular RNA from PHA/PMA-stimulated or unstimulated T-CEMB cells, Con A-stimulated or unstimulated human PBL, or CEM cells was electrophoresed through 1.2% agarose gel containing 2.2 M formaldehyde [H. Lehrach et al, Biochemistry, 16:4743

(1977)]. The formaldehyde-denatured RNA was transferred to nylon filter (Zetabind; Cuno, Meriden, CT) as described [E. M. Southern, J. Mol. Biol., 98:503-517 (1975)].

cDNA probe was made by cleaving cDNA inserts from the vector with Xho I restriction enzyme and labelled the inserts with ^{32}P using random oligonucleotides as primers in the presence of the large fragment of DNA polymerase I [A. P. Feinberg et al, Analy. Biochemistry, 132:6-13 (1983)]. The nylon filter was prehybridized for 4 hours at 43°C, hybridized with ^{32}P-labelled cDNA probe in hybridization solution consisted of 6x SSC, 0.5% SDS 5x Denhardt's solution and 100 ug/ml denatured salmon sperm DNA for 16 hours at 43°C.

After hybridization, the filter was washed two times with post-wash solution I (10 mM sodium phosphate, 0.1% SDS, 1 mM EDTA and 1 X SSC) for 30 minutes at room temperature and two times with post-wash solution II (10 mM sodium phosphate, 0.1% SDS, 1 mM EDTA and 0.2 X SSC) for 15 minutes at 68°C, dried and apply to X-ray film.

This Northern blot analysis revealed that T-cell line, CEM and T-cell hybridoma line, T-CEMB, as well as lectin-stimulated human PBL synthesized readily detectable levels of mRNA that hybridized with the MIF

clone. The messenger, however, could not be detected in RNA samples from unstimulated PBL despite prolonged exposure to film. The presence of RNA transcript in activated human PBL suggests that the human MIF gene is expressed and that MIF is the product of activated lymphocytes.

Example 5 - DNA Sequence Analysis

The nucleotide sequence of the cDNA clone of p7-1 was determined as described [G. G. Wong et al and Y. C. Yang et al, cited above] by generating ordered sets of overlapping fragments via Bal 31 nuclease digestion and subcloning into M13 vector [M. Poncz et al, Proc. Natl. Acad. Sci. USA, 79:4298-4302 (1982); and J. Messing et al, Gene, 19:269-276 (1982)]. Single-stranded DNA was prepared, and the nucleotide sequence was determined by the dideoxynucleotide chain-termination procedure [F. Sanger et al, Proc. Natl. Acad. Sci. USA, 74:5463-5467 (1977)].

Example 6 - Generation of Mutant cDNA Fragment

The relatively efficient secretion of the 12 kd protein with MIF activity from p7-1-transfected COS cells despite the lack of a clear signal peptide in the coding sequence raised the possibility that the molecularly cloned protein is not MIF but an inducer of endogenous MIF expression by the COS cells. To test this

possibility, two insertional mutations of the coding region of the p7-1 cDNA were constructed as follows.

Although the MIF cDNA clone, p7-1 contained a single Pst I site, the Pst I sites in the adapters flanking the insert rendered this site unsuitable for mutational analysis of the MIF cDNA. The two flanking Pst I sites were removed by treating the p7-1 insert that was isolated after partial digestion of the plasmid with Pst I with T4 DNA polymerase then ligating Eco RI adapters to the resulting flush ends. This adapted fragment was subcloned into the unique Eco RI site of a derivative of pXM designated pXMT4 which has no Pst I sites. A clone, p7-1-24 with the cDNA in the correct orientation was selected for mutational analysis.

Two insertional mutants of MIF were constructed. The first mutant (p7-1-24B2) was generated by inserting a 14-base oligodeoxynucleotide, 5' TGTAATTACATGCA 3', at the unique Pst I site of p7-1-24. This sequence was designed such that the MIF coding region would be interrupted by a termination codon (TAA) regardless of the orientation of insertion.

The second insertional mutant (p7-1-24232) was constructed by inserting a 99-base oligodeoxynucleotide into the Pst I site of p7-1-24. The sequence was designed to add 33 amino acids to the MIF coding region

when inserted in either orientation into the Pst I site of p7-1-24.

Clones containing the 14-base oligonucleotide or the 99-base oligonucleotide were identified by hybridization using ^{32}P-labelled 14-base oligomer or 99-base oligomer as probes. Each of these plasmids was tested for the ability to induce the secretion of the 12 kd protein observed with the original p7-1 plasmid as well as MIF activity when transfected into COS cells.

Neither the 12 kd protein nor MIF activity was detected in the supernatants from the COS cells transfected with the truncated form of MIF (p7-1-24B2). Transfection of COS cells with the mutant having the extended coding region (p7-1-24232) also failed to yield detectable levels of MIF activity or 12 kd protein. However, the COS cell supernatant was found to contain a novel species with apparent molecular weight of approximately 15,500, consistent with the expected size of the extended coding region of the insertional mutant p7-1-24232.

These experiments indicated that the induced 12 kd species from p7-1-transfected COS cells is directly encoded by the cDNA insert of p7-1 plasmid and that this protein has MIF activity.

Example 7 - MIF Biological Activities

A. MIF Assay

The MIF assay was performed according to previously described procedures [W. Y. Weiser et al, cited above; and J. T. Harrington et al, J. Immunol., 110:752 (1973)], employing human peripheral blood monocytes as indicator cells in an agarose droplet assay system.

Human peripheral blood mononuclear cells obtained by Ficoll-Hypaque centrifugation were washed two times with buffered salt solution (HBSS) and mixed with 0.2% agarose in minimal essential medium (MEM). One microliter of cells in the agarose mixture was dispensed into the center of microtiter wells using a 50 ul repeating dispenser (Hamilton Co., Reno, Nev). Samples to be tested were added to wells at 100 ul per well. The size of each agarose droplet was measured. After overnight incubation at 37°C, the total area of migration including the original agarose droplet was again measured.

The area of migration was calculated by the formula where migration = (diameter of total area/diameter of agarose droplet)^2-1. The percentage of inhibition (% I) of each sample was derived as follows:

% I = 100-(Average migration of test samples/Average migration of control samples)x100. Inhibition of 20% or greater was considered to be significant [W. Y. Weiser et al, cited above].

B. MIF Enhancement of Macrophage IL-1β and HLA-DR Gene Expression

Monocyte derived macrophages obtained by seven days culture of isolated blood monocytes were incubated with crude recombinant MIF of the present invention or supernatant from mock-transfected cells for 6 or 24 hours. Total RNA from these cells was extracted according to the method of Chirgwin et al, Biochem., 18:5294 (1979), and size fractionated by electrophoresis through 1.2% agarose gels containing 2.2M formaldehyde. The RNA was transferred to nylon filters. cDNA inserts of IL-1-beta and HLA-DR were cleaved from their respective vectors, isolated, radio-labelled with ^{32}P by random priming [Feinberg et al, Analy. Biochem., 132:6 (1983)] and hybridized with the filters.

The rMIF supernatants, but not the mock supernatants, induced IL-1-beta mRNA expression. rMIF of this invention induced a rapid and long-lasting increase of HLA-DR mRNA, whereas macrophages incubated with mock supernatant demonstrated no enhanced level of HLA-DR mRNA.

C. Activation of Human Macrophages to Release Hydrogen Peroxide

The capacity to release hydrogen peroxide is a close biochemical correlate of macrophage activation due to the prominent involvement of reactive oxygen intermediates in their anti-microbial function [See, e.g., Nathan, Trans. R. Soc. Trop. Med. Hyg., 77:620 (1983)]. The ability of rMIF to activate macrophages for hydrogen peroxide was determined as follows.

Human monocyte derived macrophages were incubated for approximately 48 hours in medium containing 2 to 1000 fold diluted rMIF supernatant of this invention or mock supernatant. Triggered by PMA, hydrogen peroxide release from these cells was measured by oxidation of scopoletin in the presence of horseradish peroxidase.

Macrophages incubated with 2, 20 and 50 fold diluted rMIF supernatant, but not mock supernatant, showed 2 to 3.6 fold enhanced release of hydrogen peroxide.

D. MIF Activation of Macrophage Leishmania donovani Killing

To determine whether rMIF of this invention could induce killing of the intracellular parasite, L. donovani, which is known to be sensitive to oxygen-dependent killing mechanisms [Murray et al, J. Clin.

Invest., 72:32 (1983)], the following assay was conducted.

Monocyte derived macrophages were incubated with rMIF supernatant for approximately 48 hours. Promastigotes of L. donovani at a parasite to cell ratio of 10:1 were added to the incubated cells. The number of intracellular parasites/100 macrophages at 2, 24, 48, and 72 hours post infection were enumerated in stained preparations of coverslips.

An increase of greater than about 22% in antileishmanial capability of cells treated with rMIF of this invention was observed. Parallel experiments combining rMIF with interferon gamma are expected to produced enhanced killing of this parasite.

Example 8. Expression of Recombinant Human MIF

To produce MIF, the cDNA encoding it is transferred into an appropriate expression vector, of which numerous types are known in the art for mammalian, insect, yeast, fungal and bacterial expression, by standard molecular biology techniques. One such vector for mammalian cells is pXM [Y. C. Yang et al, Cell, 47:3-10 (1986)]. This vector contains the SV40 origin of replication and enhancer, the adenovirus major late promoter, a cDNA copy of the adenovirus tripartite leader sequence, a small hybrid intervening sequence, an SV40

polyadenylation signal and the adenovirus VA I gene, in appropriate relationships to direct the high level expression of the desired cDNA in mammalian cells [See, e.g., Kaufman, Proc. Natl. Acad. Sci. USA, 82:689-693 (1985)]. The pXM vector is linearized with the endonuclease enzyme XhoI and subsequently ligated in equimolar amount separately to the cDNA encoding MIF that was previously modified by addition of synthetic oligonucleotides [Collaborative Research, Lexington, MA] that generate Xho I complementary ends to generate constructs for expression. These constructs can be expressed in various hosts with appropriate vectors.

a. Mammalian Cell Expression

To obtain expression of the MIF protein for use in the assay described below, the pXM construct containing the cDNA for MIF is transfected into COS cells, as described in Example 5. The conditioned medium from the transfected COS cells contains MIF biological activity as measured in the MIF assay.

The mammalian cell expression vectors described herein may be synthesized by techniques well known to those skilled in this art. The components of the vectors, e.g. replicons, selection genes, enhancers, promoters, and the like, may be obtained from natural sources or synthesized by known procedures. See, Kaufman

et al, J. Mol. Biol., 159:511-521 (1982); and Kaufman, Proc. Natl. Acad. Sci., USA, 82:689-693 (1985). Exemplary mammalian host cells include particularly primate cell lines and rodent cell lines, including transformed cell lines. Normal diploid cells, cell strains derived from in vitro culture of primary tissue, as well as primary explants, are also suitable. Candidate cells need not be genotypically deficient in the selection gene so long as the selection gene is dominantly acting. For stable integration of the vector DNAs, and for subsequent amplification of the integrated vector DNAs, both by conventional methods, CHO cells may be employed. Alternatively, the vector DNA may include all or part of the bovine papilloma virus genome [Lusky et al, Cell, 36:391-401 (1984)] and be carried in cell lines such as C127 mouse cells as a stable episomal element. Other suitable mammalian cell lines include but are not limited to, HeLa, COS-1 monkey cells, mouse L-929 cells, 3T3 lines derived from Swiss, Balb-c or NIH mice, BHK or HaK hamster cell lines.

Stable transformants are then screened for expression of the product by standard immunological, biological or enzymatic assays. The presence of the DNA and mRNA encoding the MIF polypeptides may be detected by standard procedures such as Southern blotting and RNA

blotting. Transient expression of the DNA encoding the polypeptides during the several days after introduction of the expression vector DNA into suitable host cells, such as COS-1 monkey cells, is measured without selection by activity or immunologic assay of the proteins in the culture medium.

One skilled in the art can also construct other mammalian expression vectors comparable to the pXM vector by, e.g., inserting the DNA sequences of MIF from the plasmids with appropriate enzymes and employing well-known recombinant genetic engineering techniques and other known vectors, such as pJL3 and pJL4 [Gough et al., EMBO J., 4:645-653 (1985)] and pMT2 (starting with pMT2-VWF, ATCC #67122; see PCT application PCT/US87/00033). The transformation of the vectors with MIF into appropriate host cells can result in expression of the MIF polypeptides.

b. Bacterial Expression Systems

Similarly, one skilled in the art could manipulate the sequences encoding MIF by eliminating any mammalian regulatory sequences flanking the coding sequences and inserting bacterial regulatory sequences to create bacterial vectors for intracellular or extracellular expression of MIF of the invention by bacterial cells. The DNA encoding MIF may be further

modified to contain different codons to optimize bacterial expression as is known in the art. Preferably the sequence encoding the mature MIF is operatively linked in-frame to nucleotide sequences encoding a secretory leader polypeptide permitting bacterial expression, secretion and processing of the mature MIF polypeptide, also by methods known in the art. The expression of MIF in E. coli using such secretion systems is expected to result in the secretion of the active polypeptide.

The compounds expressed through either route in bacterial host cells may then be recovered, purified, and/or characterized with respect to physicochemical, biochemical and/or clinical parameters, all by known methods.

c. Insect or Yeast Cell Expression

Similar manipulations can be performed for the construction of an insect vector for expression of MIF polypeptides in insect cells [See, e.g., procedures described in published European patent application 155,476]. The MIF cDNA will be expressed in insect cells.

Similarly yeast vectors are constructed employing yeast regulatory sequences to express the cDNA encoding MIF in yeast cells to yield secreted

extracellular active MIF. [See, e.g., procedures described in published PCT application WO 86/00639 and European patent application EP 123,289.]

Example 9. Construction of CHO Cell Lines Expressing High Levels of MIF

One method for producing high levels of the MIF protein of the invention from mammalian cells involves the construction of cells containing multiple copies of the cDNA encoding MIF.

The cDNA is co-transfected with an amplifiable marker, e.g., the DHFR gene for which cells containing increasing concentrations of methotrexate (MTX) according to the procedures of Kaufman and Sharp, J. Mol. Biol., (1982) supra. This approach can be employed with a number of different cell types.

For example, the pXM vector containing the MIF gene in operative association with other plasmid sequences enabling expression thereof is introduced into DHFR-deficient CHO cells, DUKX-BII, along with a DHFR expression plasmid such as pAdD26SVpA3 [Kaufman, Proc. Natl. Acad. Sci. USA, 82:689-693 (1985)] by calcium phosphate coprecipitation and transfection. DHFR expressing transformants are selected for growth in alpha media with dialyzed fetal calf serum. Transformants are checked for expression of MIF by bioassay, immunoassay or

RNA blotting and positive pools are subsequently selected for amplification by growth in increasing concentrations of MTX (sequential steps in 0.02, 0.2, 1.0 and 5uM MTX) as described in Kaufman et al., Mol. Cell Biol., 5:1750 (1983). The amplified lines are cloned, and MIF protein expression is monitored by the MIF assay. MIF expression is expected to increase with increasing levels of MTX resistance.

In any of the expression systems described above, the resulting cell lines can be further amplified by appropriate drug selection, resulting cell lines recloned and the level of expression assessed using the MIF assay described herein.

Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention are believed to be encompassed in the scope of the claims appended hereto.

WHAT IS CLAIMED IS:

1. A human macrophage migration inhibitory factor protein substantially free from association with other proteinaceous materials.

2. The protein according to claim 1 comprising all or a portion of the same or substantially the same amino acid sequence as follows:

M P M F I V N T N V P R A S V P D G F
L S E L T Q Q L A Q A T G K P P Q Y I
A V H V V P D Q L M A F G/G S S E P C
A L C S L H S I G K I G G A Q N R S Y
S K L L C G L L A E R L R I S P D R V
Y I N Y Y D M N A A S V G W N N S T F
A, or fragments thereof.

3. The protein according to claim 1 encoded by all or a portion of the same or substantially the same DNA as follows:

CTCGAGCTGCAGAGCTGCCTCTGCGCGGGTCTCCTGGTCCTTCTGCCATCATGCCGA
TGTTCATCGTAAACACCAACGTGCCCCGCGCCTCCGTGCCGGACGGGTTCCTCTCCG
AGCTCACCCAGCAGCTGGCGCAGGCCACCGGCAAGCCCCCCCAGTACATCGCGGTGC
ACGTGGTCCCGGACCAGCTCATGGCCTTCGGCGGCTCCAGCGAGCCGTGCGCGCTCT

GCAGCCTGCACAGCATCGGCAAGATCGGCGGCGCGCAGAACCGCTCCTACAGCAAGC
TGCTGTGCGGCCTGCTGGCCGAGCGCCTGCGCATCAGCCCGGACAGGGTCTACATCA
ACTATTACGACATGAACGCGGCCAGTGTGGGCTGGAACAACTCCACCTTCGCCTAAG
AGCCGCAGGGACCCACGCTGTCTGCGCTGGCTCCACCCGGGAACCCGCCGCACGCTG
TGTTCTAGGCCCGCCCACCCCAACCTTCTGGTGGGGAGAAATAAACGGTTTAGAGAC
AGCTCTGCAG,

a fragment thereof, or a DNA sequence capable of hybridizing thereto.

4. The protein according to claim 1 having one or more of the following characteristics:

(1) an apparent molecular weight under reducing conditions on SDS-PAGE of approximately 12 kd;

(2) biological activity in a MIF bioassay of greater than 20% inhibition;

(3) biological activity in enhancing IL-1 beta and HLA-DR gene expression in macrophages;

(4) biological activity in activating macrophage killing of Leishmania donovani.

5. The protein according to claim 1 produced by culturing a cell line transformed with a DNA sequence encoding expression of MIF in operative association with an expression control sequence therefor.

6. A process for producing MIF or a fragment thereof comprising culturing a cell line transformed with a DNA sequence encoding expression of MIF or a fragment thereof in operative association with an expression control sequence therefor.

7. A DNA sequence coding for MIF comprising a sequence of nucleotide bases the same or substantially the same as follows:

CTCGAGCTGCAGAGCTGCCTCTGCGCGGGTCTCCTGGTCCTTCTGCCATCATGCCGA
TGTTCATCGTAAACACCAACGTGCCCCGCGCCTCCGTGCCGGACGGGTTCCTCTCCG
AGCTCACCCAGCAGCTGGCGCAGGCCACCGGCAAGCCCCCCCAGTACATCGCGGTGC
ACGTGGTCCCGGACCAGCTCATGGCCTTCGGCGGCTCCAGCGAGCCGTGCGCGCTCT
GCAGCCTGCACAGCATCGGCAAGATCGGCGGCGCGCAGAACCGCTCCTACAGCAAGC
TGCTGTGCGGCCTGCTGGCCGAGCGCCTGCGCATCAGCCCGGACAGGGTCTACATCA
ACTATTACGACATGAACGCGGCCAGTGTGGGCTGGAACAACTCCACCTTCGCCTAAG
AGCCGCAGGGACCCACGCTGTCTGCGCTGGCTCCACCCGGGAACCCGCCGCACGCTG
TGTTCTAGGCCCGCCCACCCCAACCTTCTGGTGGGGAGAAATAAACGGTTTAGAGAC
AGCTCTGCAG,

a fragment thereof, or a DNA sequence capable of hybridizing thereto.

8. A cell transformed with a DNA sequence of claim 7 in operative association with an expression control sequence.

9. The cell according to claim 8 comprising a mammalian or bacterial cell.

10. Homogeneous MIF having a biological activity in the MIF assay of greater than 20% inhibition.

11. A pharmaceutical composition comprising a therapeutically effective amount of MIF or a biologically active fragment thereof in a pharmaceutically effective vehicle.

12. The composition according to claim 11 further comprising therapeutically effective amounts of an additional cytokine, hematopoietin, growth factor or tumor-activated antibody.

13. The composition according to claim 12 where said cytokine is selected from the group consisting of IL-1, IL-2, IL-3, IL-4, IL-6, GM-CSF, G-CSF, M-CSF, the interferons, erythropoietin.

14. The composition according to claim 13 wherein said additional cytokine is IFN-gamma, M-CSF or GM-CSF.

15. A plasmid vector comprising a DNA sequence of claim 7.

16. A method for treating cancer comprising administering to a patient an effective amount of MIF or a fragment thereof.

17. A method for treating infection comprising administering to a patient an effective amount of MIF or a fragment thereof.

18. A method for enhancing wound healing comprising administering to a patient an effective amount of MIF or a fragment thereof.

19. A method for stimulating the immune system comprising administering to a patient an effective amount of MIF or a fragment thereof.

20. A method for enhancing the effectiveness of a vaccine comprising administering said vaccine following or simultaneously with a therapeutic composition comprising MIF.

21. The method according to claim 19 further comprising administering simultaneously or sequentially with said MIF an effective amount of at least one hematopoietin, cytokine, growth factor or antibody.

22. The method according to claim 21 wherein said cytokine is M-CSF, GM-CSF or IFN-gamma.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/01355

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 07 K 13/00, C 12 P 21/02, C 12 N 15/00, A 61 K 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 07 K, C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	EP, A, 0162812 (CIBA-GEIGY AG) 27 November 1985 see claim 1	1
	--	
Y	EP, A, 0263072 (CIBA-GEIGY AG) 6 April 1988 see claims 1-3,21-34	1-15
	--	
Y	Cellular Immunology, vol. 90, 1985, 1985, Academic Press, Inc., (New York, US), W.Y. Weiser et al.: "Generation of human hybridomas producing migration inhibitory factor (MIF) and of murine hybridomas secreting monoclonal antibodies to human MIF", pages 167-178, see the whole article, especially	1-15

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
14th June 1990	13. 07. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. PEIS

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SEC ND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	page 176, table 3 (cited in the application) --	
P,X	Proceedings of the National Academy of Sciences USA, vol. 86, October 1989, (Washington, D.C., US), W.Y. Weiser et al.: "Molecular cloning of a cDNA encoding a human macrophage migration inhibitory factor", pages 7522-7526, see the whole article, especially page 7525, figure 3 ----------	1

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9001355 SA 35384

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 06/07/90
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0162812	27-11-85	AU-B- 592753	25-01-90
		AU-A- 4281385	28-11-85
		JP-A- 60248617	09-12-85
EP-A- 0263072	06-04-88	AU-A- 7930987	19-05-88
		JP-A- 63157997	30-06-88